

Original Article

IN VITRO EVALUATION OF THE ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTIPROLIFERATIVE ACTIVITIES OF THE LEAF EXTRACTS OF *EXCOECARIA AGALLOCHA* L.

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ABSTRACT

Objective: To evaluate the antioxidant, anti-inflammatory and antiproliferative activities of the leaf extracts of *Excoecaria agallocha* L.

Methods: To estimate the antioxidant effect of the extract from *Excoecaria agallocha*, various assays were carried out. Estimation of total phenolics was done by Folin-Ciocalteu micro-method, total antioxidant capacity was determined by sulphuric acid-ammonium molybdate method, reducing power assay was carried out by FRAP (Ferric cyanide (Fe³⁺) Reducing Antioxidant Power) assay method and free radical-scavenging activity was estimated by DPPH (diphenyl-picrylhydrazine) and ABTS methods. Bovine serum denaturation inhibition assay was performed to test the *in vitro* anti-inflammatory activity of the extract, MTT assay was carried out to find out the antiproliferative effect of the extract against SKMEL 28 skin cancer cell lines. The crude ethyl acetate fraction was finally analyzed by thin layer chromatography to reveal 8 bands indicating presence of various compounds that probably contributed to the various biological activities.

Results: The total antioxidant activity, total phenolics content and reducing power assay tested with various solvent fractions of *Excoecaria agallocha* leaf extract showed that ethyl acetate fraction had the highest antioxidant activity, reducing power and phenolics content among other fractions. The scavenging effect of extracts and standards on ABTS^{•+} decreased in the order: ethyl acetate > n butanol > water > petroleum ether > gallic acid. The relative percentage of inhibition of BSA denaturation by the ethyl acetate extract was found to be 85.38% at a concentration of 100 µg/ml in comparison to the standard diclofenac sodium which brought about 13.65% of inhibition of BSA denaturation at the same concentration. The G₁₅₀ was found to be 19.02 µg/ml for DMSO and 39.2 µg/ml for ethyl acetate fraction against SKMEL 28 skin cancer cell lines.

Conclusion: These results suggest that the extracts of *Excoecaria agallocha* possess significant antioxidant, anti-inflammatory and antiproliferative activities.

Keywords: *Excoecaria agallocha*, Antioxidant, Anti-inflammatory, Bovine serum albumin (BSA) denaturation inhibition, Antiproliferative, TLC

INTRODUCTION

The traditional, complementary and alternate systems of treatments rely on the vast amount of bioactive compounds present in plants with high potentials to cure a vast variety of diseases including cancer. More than half of the currently available drugs are derived from plants or are compounds synthesized/ designed with a natural compound as a model/lead molecule. Molecular modifications of this type generate structural analogues with greater pharmacological activity and fewer side effects. Thus, more affordable drugs which are far more efficient than the present ones are obtained [1].

Excoecaria agallocha L. (Family Euphorbiaceae) is a mangrove plant extensively studied for its chemical composition [2-6]. The plant is generally called blind-your-eye mangrove or buta-buta. It is known by various names in the Indian sub-continent. In south India, it is known as Gangiva, Tejbala (Hindi), Thillai (Tamil) and Koomatti (Malayalam) [7]. It is the only mangrove species with milky latex. Studies on the wound healing property of the plant extract demonstrated that it was comparable with that of the standard medicine, furacin ointment [8]. The antioxidant [9-11], antibacterial [11, 13, 14], antifungal [15], anti-inflammatory [16], anticancer [17, 18], antifilarial [19] and antiviral [20] properties of the plant are well documented. The previous studies revealed antifungal [21] and antibacterial [22] activity which could be attributed to the presence of secondary metabolites [23]. It is clear that *Excoecaria agallocha* is rich in secondary metabolites but there are no reports as yet on the details of the possible role of these secondary metabolites as antioxidants and also on the antiproliferative activity of the extract. The present study is an effort in this direction and perhaps is the first of its kind in that aspect. The anti-inflammatory effect of the extract was also checked so as to find a lead in the treatment of such cancer cases where inflammation is predominant.

MATERIALS AND METHODS

Materials

Analytical reagent grade chemicals purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) were used throughout unless stated otherwise. Water used was purified with a Milli-Q system (Millipore, Bedford, MA).

Plant material

The leaves of *Excoecaria agallocha* was collected during March-April season from Wadakara, near Kozhikode, Kerala, India. Taxonomical identification of the plant was done by Dr. P. M. Unnikrishnan, FRLHT, Bangalore, India.

Extract preparation

The leaves (40 g) were shade-dried for 7 d and ground coarsely and extracted with methanol (3 times, 500 ml for 2 d) at room temperature. The extracts were combined and concentrated in vacuum at 30 °C. The residue (12.5 g) was diluted with water (~ 50 ml) and partitioned against petroleum ether (PE), ethyl acetate (EA) and n-butanol (nB) and each were concentrated to dryness. The yield of various fractions was noted. All the fractions were reconstituted in minimum quantity of the respective solvents and stored in aliquots for further analysis. To check the antioxidant potential of the extracts, the various fractions were subjected to different assays as detailed below.

Antioxidant assays

Estimation of total phenolics

To determine the total phenolic content of the extract, the Folin-Ciocalteu micro-method as given by [24] was followed. Briefly, 20 µl of extract solution were mixed with 1.16 ml distilled water and 100

μl of Folin-Ciocalteu reagent, followed by addition of 300 μl of Na_2CO_3 solution (20%) after 1 min and before 8 min. Subsequently, the mixture was incubated in a shaking incubator at 40 °C for 30 min and its absorbance was measured at 760 nm. Gallic acid was used as the standard for calibration curve. The phenolic content was expressed as gallic acid equivalents using the linear equation based on the calibration curve.

Total antioxidant capacity

An aliquot of 0.1 ml of sample solution (1 mg/ml in methanol) was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After the samples had cooled down to room temperature, the absorbance was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent was used for the sample. This was incubated under the same conditions as the rest of the samples. The antioxidant activity of extracts was expressed as equivalents of ascorbic acid which served as the standard. The % total antioxidant activity (TAC) was calculated as.

$$\% \text{ TAC} = (A_s - A_c) / (A_{aa} - A_c) \times 100$$

Where A_c is the absorbance of the control (blank, without extract),

A_s the absorbance in the presence of the extract and

A_{aa} the absorbance of ascorbic acid.

Reducing power assay (Ferric cyanide (Fe^{3+}) reducing antioxidant power assay-FRAP method)

The ability of extracts to reduce iron (III) was assessed by the reducing power assay carried out according to Oyaizu [25]. The dried extract (125–1000 μg) in 1 ml of the corresponding solvent was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$; 10 g/l), then the mixture was incubated at 50 °C for 30 min. After incubation, 2.5 ml of 10% trichloroacetic acid (100 g/l) was added and the mixture was centrifuged at 1650xg for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl_3 (1g/l) and the absorbance was measured at 700 nm. The reduction of ferric cyanide (Fe^{3+}) (present in the reaction mixture) to ferrous cyanide (Fe^{2+}) by the extracts results in the development of dark color depending on the reducing capacity of the extract and thereby higher absorbance. High absorbance indicates high reducing power. Ascorbic acid was taken as the standard.

Free radical-scavenging activity using DPPH

The antioxidant activity of extract and Ascorbic acid was measured in terms of electron transfer/hydrogen donating ability, using the DPPH radical method of [26] modified by [27]. The extract at various concentrations was added to 3.9 ml (0.025 g/l) of DPPH radical solution. The decrease in absorbance at 515 nm was determined continuously at every 1 min with a UV-Visible Spectrophotometer. The amounts of extract and standard required to reduce the initial OD value of DPPH radical to half is recorded as the IC_{50} value.

Free radical-scavenging activity using ABTS

ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM ammonium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5; this will result in incomplete oxidation of the ABTS [28]. Prior to assay, the solution was diluted in ethanol (about 1:88 v/v) to give an absorbance of 0.700 ± 0.02 at 734 nm in a 1 cm cuvette and equilibrated to 30 °C, the temperature at which all the assays were performed. The stock solution of extract and gallic acid in methanol were diluted such that after introduction of a 10 μl aliquot of each dilution into the assay, they produced 50% inhibition of the blank absorbance. After the addition of 1.0 ml of diluted ABTS radical solution to 10 μl of antioxidant compounds or standards (final concentration 0–15 M, prepared in methanol), it was

incubated at 30 °C exactly for 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay.

Based on the results of various antioxidant assays, the fraction that had highest antioxidant potential was further checked for anti-inflammatory activity and antiproliferative activity. Bovine serum denaturation inhibition assay was carried out to test the anti-inflammatory activity and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was used to check the effect of the extract on the viability of SKMEL 28 skin cancer cell lines. Column chromatographic separation of the fraction that had highest antioxidant potential and further TLC analysis was also performed.

In vitro anti-inflammatory assays

Generally, various *in vitro* assays are conducted to get a preliminary idea of the anti-inflammatory effect of plant extracts. Bovine serum denaturation inhibition assay is one such assay to detect the preliminary anti-inflammatory activity. The assay was conducted with that fraction of *E. agallocha* L. which had highest antioxidant potential to check its ability to inhibit the denaturation of BSA.

BSA denaturation inhibition assay

The ability of the fraction of *E. agallocha* towards inhibition of albumin denaturation was checked according to [29] with slight modification. In short, the fraction was dried in vacuum oven and re-dissolved in iso saline. Different concentrations of the fractions were made and added to 1.8 ml of 1 % BSA solution. The pH was adjusted to 6.5 using 1N HCl and the solution was incubated at 37 °C for 20 min and then heated to 57 °C for 10 min. After cooling, the absorbance was measured at 660 nm. Diclofenac sodium was used as the standard and a solution without sample/extract was considered as the control. The experiment was done in duplicates. The relative percentage inhibition of protein denaturation was calculated as.

$$\text{Relative \% of inhibitory activity} = (A - A_{\min}) / (A_{\max} - A_{\min}) \times 100$$

Where A-absorbance of sample

A_{\min} -absorbance of control

A_{\max} -highest absorbance of standard

Cell proliferation assay

The standard MTT assay was carried out to find out the antiproliferative effect of the fraction of *E. agallocha* L. on SKMEL 28 skin cancer cell lines following the protocol of [30] with slight modification.

Cell culture

SKMEL 28 skin cancer cell lines (a type of melanoma cell line) were purchased from NCCS, Pune and was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (*In vitro* gen) and grown to confluency at 37 °C in 5 % CO_2 in a humidified atmosphere in a CO_2 incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized with 500 μl of 0.025 % Trypsin in PBS/0.5 mM EDTA solution (Himedia) for 2 min and passaged to T flasks in complete aseptic conditions. DMSO and extracts were added to grown cells at a final concentration of 10 $\mu\text{g}/\text{ml}$, 20 $\mu\text{g}/\text{ml}$ and 40 $\mu\text{g}/\text{ml}$ and incubated for 24 h. The per cent viability was determined by the standard MTT assay after 24 h of incubation.

Cell viability assay using MTT

MTT is the cell viability assay followed globally to test the ability of any compound to induce toxicity in cell lines. It is a colorimetric assay to detect the IC with GI value of any drug. The assay is based on the principle that viable cells converted MTT to formazan crystals, which generated a blue-purple color that can be read colorimetrically.

In short, cells (1×10^4 per 200 μl medium) were cultured in 96 well culture plates and incubated with different concentrations of DMSO and the extract for 24 h. 25 μl of 5 mg/ml MTT were added to the cells and again incubated for 4 h at 37 °C. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product (formed as a result of the

reduction of MTT by mitochondrial succinate dehydrogenase). The supernatant was removed and the insoluble formazan crystals were dissolved in 100 µl DMSO. The absorbance was measured at 570 nm using a microplate reader (ELISASCAN, ERBA). Since reduction of MTT can only occur in metabolically active cells, the level of activity is a direct measure of the viability of the cells. The percentage of viable cells after treatment with the extract was calculated as

$$\% \text{ viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \times 100$$

The minimum concentration of extract required to cause 50% inhibition of cell growth (GI_{50}) was also calculated from this.

Further fractionation and TLC analysis

The fraction with the highest antioxidant potential was further subjected to column chromatographic separation. The solvent was removed from the extract by vacuum evaporation and was separated by column chromatography on a silica gel (SiO₂, 100–200 mesh-PE/AcOEt 90: 10, 80: 20, 70: 30, 60: 40, 50: 50). The fractions obtained from each were combined and concentrated to load on TLC plate and developed. The results were analyzed and reported.

Statistical analysis

Data represented are collected from three independent experiments performed in triplicates and expressed as mean±standard error (±SD).

RESULTS

Crude extract and fractions were obtained as described in materials and methods. To start with, 31.32 % of crude extract was obtained from 40 g of dry leaves. The dry weights of the concentrated fractions after fractionation were 1.58 g (PE), 1.2g (Ethyl acetate), 1.525 g (n Butanol) and 1.56 g (water).

Antioxidant assays

Total antioxidant activity, total phenolics and reducing power

The antioxidant studies analyzed in terms of the total antioxidant activity, total phenolics and reducing power assays revealed excellent results as depicted in table. Among the various extracts tested, ethyl acetate fraction gave the highest activity in all the assays. The most significant among these results was the phenolic content of ethyl acetate fraction represented in terms of gallic acid equivalents. The results of the various assays are given in table 1.

Table 1: Antioxidant activity analysis of the extracts of *E. agallocha* L. Values are expressed as mean±SD of three replicate (n=3)

Extracts	Total antioxidant activity (mg ascorbic acid eq./g extract)±SD	Total phenolics (mg gallic acid eq./g extract)±SD	Reducing power (mg ascorbic acid eq./g extract)±SD
Petroleum Ether	255.37±0.4	70.25±0.04	26.90±0.03
Ethyl acetate	399.85±0.87	691.44±0.48	299.86±0.07
n-Butanol	66.46±0.36	325.86±0.04	220.63±0.08
Water	11.42±0.15	455.37±0.20	42.36±0.03

Free radical-scavenging activity

DPPH is most widely used to evaluate the free radical scavenging efficacy of a wide range of antioxidants present in plant extracts. Generally, the hydrogen donating antioxidants present in the extract will reduce the DPPH radical in alcohol solution to form the yellow colored diphenyl-picrylhydrazine [31]. The level of free radical scavenging activity of various antioxidant compounds present in *E. agallocha* was evaluated in terms of the DPPH radical reduced. The preliminary antioxidant assay of the various extract from *E. agallocha* revealed excellent results. The EC_{50} for different extracts are represented in fig. 1. The higher the antioxidant potential, lower will be the concentration required for reduction.

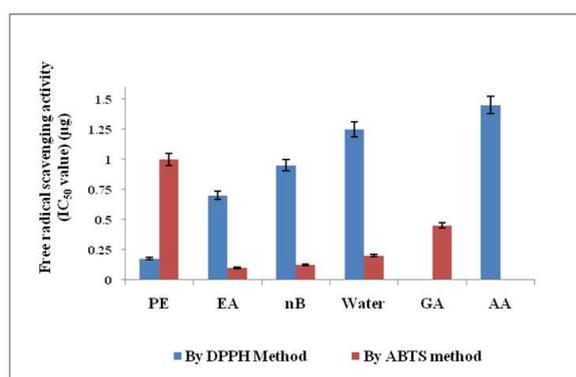


Fig. 1: Free radical scavenging activity of various extracts of *Excoecaria agallocha* L. by DPPH and ABTS methods (PE-petroleum ether, EA-ethyl acetate, nB-n-butanol, GA-gallic acid, AA-ascorbic acid) (Data represents the results of experiments done in triplicates±SD, n=3)

Pre-formed ABTS radical was reduced by the antioxidants present in the extract. After proper incubation time, the ABTS•+remaining in the test solution was measured spectrophotometrically at 734 nm.

Most of the extracts tested exhibited very significant and efficient ABTS•+radical scavenging activity in a concentration dependent manner. As observed from the results of antioxidant assay as in table 1, the extracts showed competent free radical scavenging activity when compared to the standard. The scavenging effect of extracts and standards on ABTS•+decreased in the order: EA>n Butanol>Water>PE>Gallic acid. The EC_{50} values of the various extracts are represented in the table.

As revealed by various antioxidant and free radical scavenging activity assays conducted with the various fractions of *E. agallocha*, it was observed that the ethyl acetate fraction had the maximum antioxidant activity. Based on this, further analyses were carried out with this fraction. The ethyl acetate fraction was subjected to preliminary anti-inflammatory and antiproliferative assays and was also further separated by column chromatography. The chromatographic fractions were analyzed by thin layer chromatography.

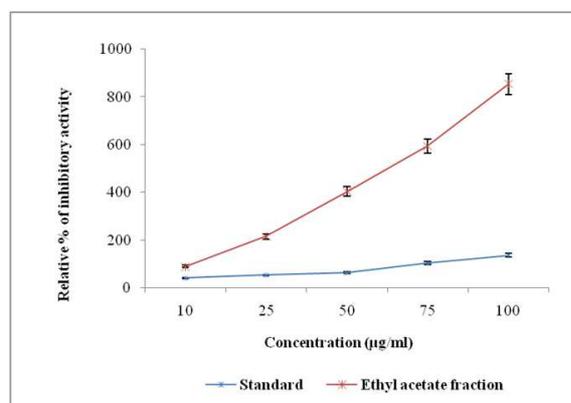


Fig. 2: BSA denaturation inhibition assay. The capacity of the extract to protect BSA from denaturation was found to be almost six times more than the standard anti-inflammatory drug, diclofenac sodium. (Data represents the results of experiments done in triplicates±SD, n=3)

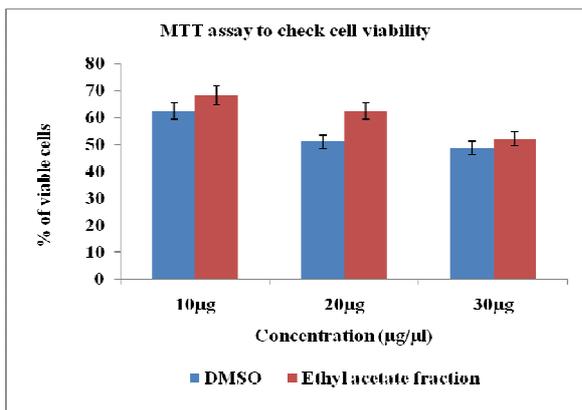


Fig. 3: MTT assay to determine the percentage of viable SKMEL 28 skin cancer cell lines. (Data represents the results of experiments done in triplicates±SD, n=3)

In vitro anti-inflammatory activity

The anti-inflammatory activity of ethyl acetate fraction tested in terms of the inhibition of BSA denaturation revealed that the extract was capable of inhibiting protein denaturation in a concentration dependent manner (fig. 2). The relative percentage of inhibition of

BSA denaturation by the extract was found to be 85.38% at a concentration of 100 µg/ml, whereas the standard diclofenac sodium brought about 13.65% of inhibition of BSA denaturation at the same concentration. The capacity of the extract to protect BSA from denaturation was found to be almost six times more than the standard anti-inflammatory drug, diclofenac sodium. This sort of inhibition of BSA denaturation is a typical feature of anti-inflammatory compounds.

Cell proliferation assay

MTT assay, carried out to determine the antiproliferative capacity of the ethyl acetate extract, revealed that the extract was capable of inhibiting the growth of SKMEL 28 skin cancer cell lines in a concentration dependent manner (fig. 3). The GI₅₀ was found to be 19.02 µg/ml for DMSO and 39.2 µg/ml for ethyl acetate fraction.

DMSO and the ethyl acetate fraction when added at 10, 20 and 40 µg/ml to the cell lines gave the effect similar to apoptosis. The untreated cells (A in fig. 4) which served as control cells in the antiproliferative assay showed well-spread and confluent layer of cells whereas cells treated with different concentrations of DMSO and ethyl acetate showed varying degrees of damage. The cells were rounded off (instead of long, elongated ones as in control cells). The number of normal cells was found to be less in treated cells (DMSO at 10, 20 and 40 µg/ml concentrations represented by fig. 4 (B, C and D) and ethyl acetate at 10, 20 and 40 µg/ml concentrations represented by fig. 4 (E, F and G respectively).

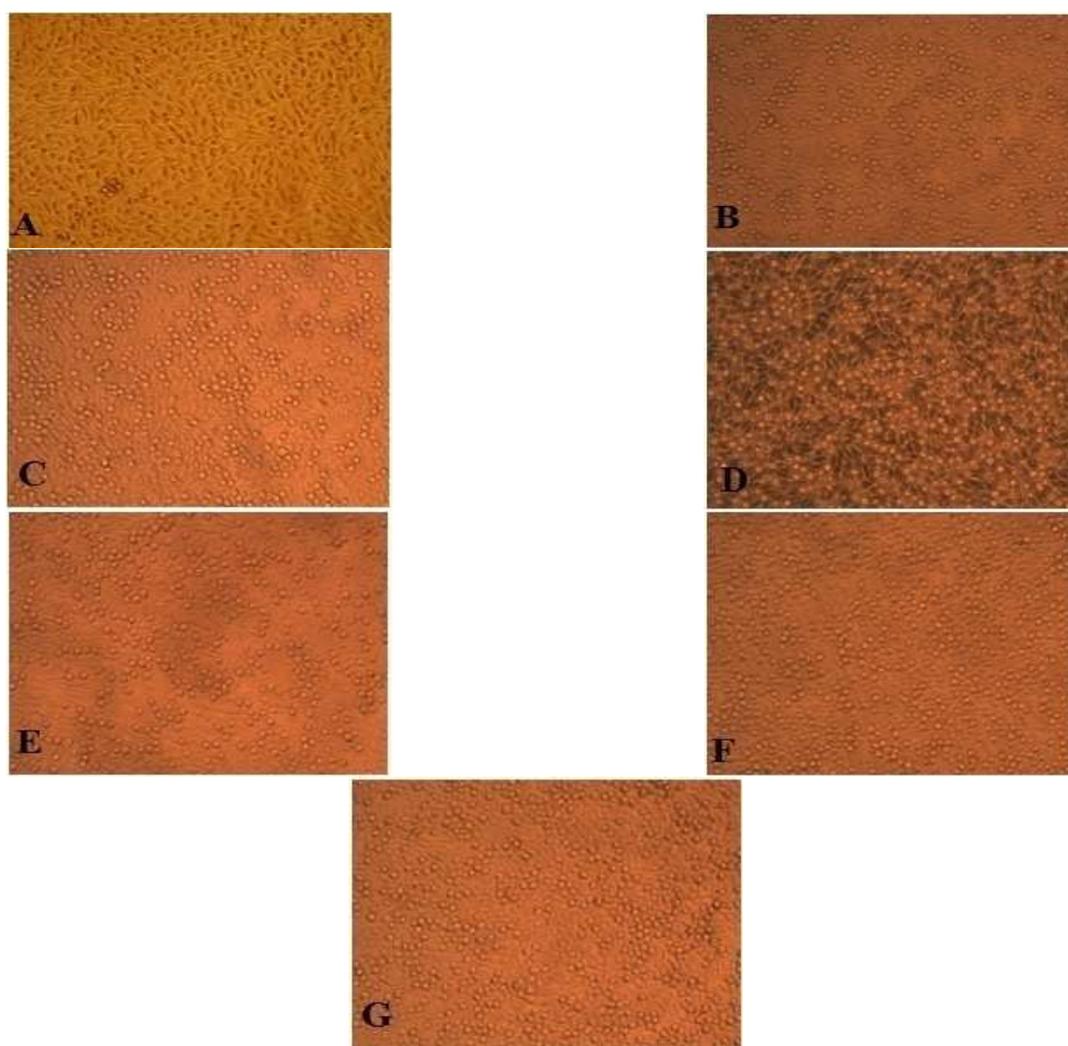


Fig. 4: Effect of DMSO and ethyl acetate fraction on proliferation of SKMEL 28 skin cancer cell lines A-Control (untreated cells), B, C, D-Cells treated with DMSO (10µg/ml, 20 µg/ml and 40 µg/ml respectively) and E, F, G-cells treated with ethyl acetate fraction of *Excoecaria agallocha* L. (10µg/ml, 20µg/ml and 40 µg/ml respectively)

Column chromatography and TLC

The active crude fraction of ethyl acetate was further subjected to chromatographic separation on a silica gel. The 70:30 (PE/AcOEt) fractions showed the maximum separation; it yielded 19 fractions which were combined and subjected to thin layer chromatographic (TLC) analysis. It revealed 8 bands (fig. 5a). The combined fraction was further subjected to chromatographic separation which yielded 12 fractions. When examined by TLC, only fraction number 4 (fig. 5b) gave a single band. This fraction was concentrated for further studies to understand the mechanism behind the antiproliferative activity. The detailed experiments in this regard are underway.

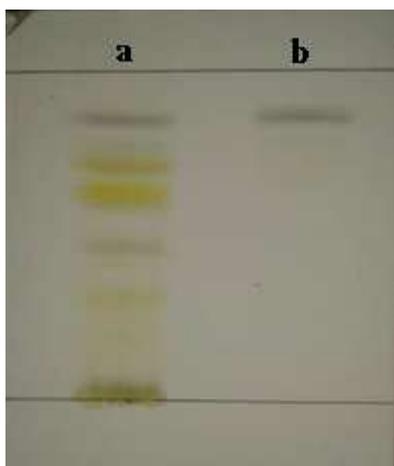


Fig. 5: Thin layer chromatography of the ethyl acetate fraction of *Excoecaria agallocha* L. The ethyl acetate fraction was subjected to chromatographic separation on silica column. The fractions were pooled and run on TLC. It revealed eight distinct bands (a). These fractions were further separated on silica column and individual fractions were again run on TLC. Fraction number four gave a single band on TLC plate (b)

DISCUSSION

The efficiency of plants and plant products in reducing the damaging effects of reactive oxygen species (ROS) is mainly due to the presence of antioxidant compounds [32]. Antioxidants are also capable of reducing the influence of ROS on chronic diseases including cardio vascular diseases and cancers.

The antioxidant and free radical scavenging activity of plants are one of the most widely and extensively studied aspects in plant and medical science. The antioxidant capacity of the bark of *E. agallocha* was studied using a series of standard assays [33]. But the details of the antioxidant assays with the leaves remained unexploited hitherto. The present study on the leaf extracts of *E. agallocha* revealed effective antioxidant and free radical scavenging activity. The antioxidant studies with bark extracts of *E. agallocha* [34] also revealed similar results where the aqueous fraction had the highest total polyphenol content, 348 mg gallic acid equivalent (GAE)/g followed by ethanol fraction which showed 160.5 mg of gallic acid equivalent activity per g. In the present study, it was found that among the various extracts tested, the ethyl acetate fraction showed better activity in terms of total antioxidant content (399.85 ± 0.87 mg gallic acid equivalents per g of the plant extract), total phenolics content (691.44 ± 0.48 mg gallic acid equivalents per g of the plant extract) and reducing power (299.86 ± 0.07 ascorbic acid equivalents per g of the plant extract). The results of radical scavenging activity by ABTS and DPPH also revealed the antioxidant potential of the plant extracts. It was especially noteworthy that the ethyl acetate fraction had more scavenging effect than the other extracts as it was observed that only small quantities of ethyl acetate fraction were required to bring about the effect compared to other extracts. This was typical as already revealed by the alkaloid rich fractions from

the leaves of *E. agallocha* [35]. Their results were comparable to that of the standard free radical scavenger butylated hydroxyl anisole (BHA). However, more studies are warranted to understand the role of antioxidants from the extracts of *E. agallocha* on the anti-inflammatory effect.

It was also observed that antioxidants can act as anti-inflammatory agents [32]. It is well known that ROS activate a number of intracellular signaling pathways thereby activating various pro-inflammatory cytokines. They can also act as secondary messengers, thereby activating the production of other inflammatory mediators [36]. Plant derived compounds such as gallic acid, rutein, vanillic acid; quercetin and kaempferol can block distinct signal transduction events necessary for the activation of such inflammatory mediators and thus can act as anti-inflammatory agents [37].

It is a well known fact that inflammation and tumor are closely related. The role of inflammation in carcinogenesis was reported by Rudolf Virchow as early as 1863 [38]. Consequently, anti-inflammatory agents can give clues on anticancer molecules. The effect of the damage of DNA or protein (caused by reactive oxygen and nitrogen species) in inflammatory disease and progression to cancer was documented earlier [39]. Thus, the interconnection between antioxidant, anti-inflammatory and anticancer extracts are to be scrutinized to understand the full potential of the extract. The *in vitro* anti-inflammatory assays carried out will undeniably facilitate to avoid animal studies especially during the early stages of research and thus could simplify animal ethical issues. In the present study, the antioxidant, anti-inflammatory and antiproliferative studies were carried out with the ethyl acetate fraction of *E. agallocha*, a mangrove plant much discussed in ancient literatures.

Free radical-induced damages may result in the denaturation of proteins [40]. Albumin is susceptible to denaturation as a result of the modifications to the amino, carboxyl or disulfide groups [41]. These types of modifications are often found to effect in inflammation [36]. Consequently, there is in effect, a strong correlation between protein damage and inflammation. Plant extracts that are capable of inhibiting free radical-induced albumin denaturation will help to establish potent anti-inflammatory activity present in the extract. In the present study, the protein denaturation inhibition studies conducted showed that the ethyl acetate fraction of *E. agallocha* was competent enough to be used as anti-inflammatory agent. The extract was almost six times more effective in inhibiting the protein denaturation than the standard drug used in the study. This is a novel finding as far as the extracts of *E. agallocha* are concerned. This result is in corroboration with that of [42] where significant anti-inflammatory activity was observed for *N. nouchali* extract with a capability of controlling the production of autoantigen to inhibit the denaturation of protein.

Melanomas are the most aggressive forms of all types of skin cancers. Although non melanoma skin cancer is generally curable, treatment of melanoma is complicated and involves a combination of surgery, chemotherapy, radiation therapy and targeted therapy [43]. SKMEL 28 skin cancer cell lines are typical melanoma cell lines that need any of the above said modes of treatment. The present study revealed the antiproliferative effect of the ethyl acetate fraction from *E. agallocha* leaves. Thus it can be said for sure that *Excoecaria agallocha* has compounds that are capable of inducing cell toxicity effect on melanoma which implies that it can be used in cosmetics apart from pharmaceutical and medical formulations for the control of melanoma and other skin cancer types.

Plant extracts are generally found to exert cell cytotoxicity or antiproliferative effects by various methods like induction of apoptosis and cell cycle arrest, interference in cell signaling pathways, etc. The exact mode of action of *Excoecaria agallocha* leaf extract in the present case against SKMEL 28 melanoma is a subject of further analysis.

The chromatographic separation and further TLC analysis of ethyl acetate fraction of *E. agallocha* revealed the presence of many compounds which could have contributed to the broad spectrum of activity represented by the fraction. Given the vast amount of data on the traditional applications of this plant, it is not surprising to find this wide range of compounds. The chromatographic

separation, ^1H NMR analysis and bioautography screening of methanol extract of *E. agallocha* was already elaborated [11] where TLC was used for checking the presence of active compounds. Further fractionation of the ethyl acetate fraction in the present study yielded 12 fractions of which one showed a single compound as revealed by TLC. More studies are needed to find out the nature of individual compounds and the activity represented by each fraction. Some of these are in progress.

Conversely, to the best of our knowledge, the present study is the first ever report of this kind which discusses the antioxidant, anti-inflammatory and antiproliferative effects of *E. agallocha* leaf extract. More studies are warranted to appreciate the organized interconnected activity of the extract; to see if the antioxidant, anti-inflammatory and antiproliferative activities are inter-related. Further studies *vis-à-vis* the effect of the extract on cell lines and its mode of action are underway.

CONCLUSION

To conclude, it could be said that the present study had added systematic evidence to the already available literature on the antioxidant, anti-inflammatory and antiproliferative effects of the extracts from *E. agallocha* L. Further studies are underway to verify and authenticate the mechanism behind the antiproliferative property of the extract. The vast amount of activity ascribed to the plant is definitely a source of inspiration to work with. It is hoped that the results of the present study will inspire the scientific community to further explore the plant in detail for its varied effects.

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ABBREVIATION

ABTS-2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, TLC-thin layer chromatography, DMSO-dimethyl sulphoxide, FRAP-Ferric cyanide (Fe^{3+}) Reducing Antioxidant Power assay, DPPH-, b.

CONFLICT OF INTERESTS

The authors disclose that there are no conflicts of interest.

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